Rhythmic motor activity and interlimb co-ordination in the developing pouch young of a wallaby (*Macropus eugenii*)

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1. The forelimb motor behaviour of developing wallaby was studied. A clock-like alternating movement was reactivated whenever the animal was removed from the pouch.

2. Forelimb stepping frequency increased during the first 3 weeks of development, while the phase relationship remained constant. Forelimb activity could be affected by altering the afferent feedback from the contralateral limb, or an increase in ambient temperature.

3. *In vitro* experiments were performed using an isolated brainstem–spinal cord preparation from animals up to 6 weeks postnatal. Fictive locomotor activity could be evoked by electrical stimulation or bath-applied NMDA (< 10 μM).

4. Bath-applied strychnine (10–25 μM) and bicuculline (10–50 μM) disrupted the phase relationship between motor pools, while rhythmic motor discharge remained in the absence of these inhibitory pathways.

5. The present findings indicate that the pattern generator that underlies the robust forelimb movement during the first journey to the pouch is retained for different motor functions during in-pouch development. The neural network that underlies such behaviour can be divided into two major components, a rhythm generator within each hemicord, and a pattern co-ordinating pathway which involve both glycinergic and GABAergic interneurones.

The tammar wallaby is a native Australian wallaby with average adult weight around 4–5 kg. Like other marsupials, this mammalian species adopts a reproduction strategy with a short intrauterinal gestation followed by a protracted in-pouch development during which a large part of the nervous system is developed. For example, lumbar motoneurone cell death takes place entirely during the postnatal period (Comans, McLennan & Mark, 1987) and eye opening does not occur until 140 days after birth. This makes the wallaby pouch young the preparation of choice for developmental and perturbation experiments since similar procedures in eutherian animals would require *in utero* manipulation (Marotte & Mark, 1988; Comans, McLennan, Mark & Hendry, 1988; Marotte, 1993). Despite the immaturity of the nervous system, each newborn wallaby must climb into the pouch and attach to the teat, unaided by the mother. Thus it must be equipped with a functional motor system and a sufficient sensory system to control the former, in order to complete this vital task. This functional motor capacity during early development also makes the wallaby a potentially useful preparation for studying the spinal central pattern generator (CPG).

It has long been observed that the spinal cord, even in the absence of higher motor centres and sensory afferents, can initiate stereotyped motor commands (see Grillner, 1975, for review). However, the operation of the mammalian spinal CPG remains unclear. Various embryonic and neonatal preparations have been established to study the CPG with the rationale that a developing nervous system, before all the components are differentiated and inter-connected, is likely to provide a less intractable model (Kahn & Roberts, 1982; O'Donovan, 1987; Kudo & Yamada, 1987). When compared with the rat, the neonatal wallaby possesses a more robust motor apparatus, while the morphology of the brachial spinal cord resembles a 15–16 day rat fetal cord (Harrison & Porter, 1992). This study utilized this unique combination to explore the neural mechanisms that underlie the mammalian spinal CPG.

The first part of the study was to document forelimb behaviour during the in-pouch period. It was of particular interest to see whether the intense alternating forelimb activity expressed during the first journey is retained after the animal has reached the pouch. Studies from lower vertebrates suggest that inhibitory mechanisms play an important role in determining the rhythmic process as well as the pattern of activity (Cohen & Harris-Warrick, 1984; Dale, 1985; Grillner, Wallen & Brodin, 1991). GABAergic and glycinergic transmissions have been shown to affect rhythmic locomotor activity in the mammalian circuits (Atsuta, Abraham, Iwahara, Garcia-Rill & Skinner, 1991; Cazalets, Sqalli-Houssaini, Clarac, 1994; Cowley & Schmidt, 1995; Bracci, Ballerini & Nistri, 1996). The second part of the study made use of the vigorous alternating behaviour of the forelimbs and the small size of the neonatal wallaby

central nervous system to determine possible inhibitory pathways that are involved using *in vitro* conditions. The results showed that isolated preparations from animals up to the 6th postnatal week could be kept viable for over 24 h, and that they express fictive locomotion that resembled forelimb behaviour of intact animals. Bath-applied strychnine and bicuculline disrupted the alternating activity and revealed a common excitatory pathway. Some of the preliminary results have appeared in abstract form (Ho, 1995).

METHODS

Wallaby colony

Wallabies (*Macropus eugenii*) were obtained from a breeding colony established by Professor R. Mark at the Australian National University. The establishment of the colony and all experimental procedures were approved by the Animal Experimentation Ethics Committee of the Australian National University. Timed pregnancy was obtained by removing a pouch young from the female. This led to the reactivation of the dormant blastocyst and birth after a 26–28 day gestation period (Renfree & Tyndale-Biscoe, 1978). The breeding season normally occurs in summer and autumn, and can be extended into winter by injection of bromocriptine (5 mg kg^{-1} I.M.; Sigma; Tyndale-Biscoe & Hinds, 1984). All animals less than 2 weeks of age were of known birth date. Older animals were either of known birth date or their age was estimated by head length measurement (distance between the occipital pole and the mid-line symphysis between the left and right maxillae), which was accurate to within $\pm$ 2 days.

Behavioural study

Motor behaviour was studied in pouch young from postnatal day 1 (P1) up to P135. The day of birth is denoted as P0. Pouch exit begins at about P200. A total of forty-eight animals were used. Some of the animals were used for other experiments after the behavioural study, and others were returned to the pouch. As animals were not endothermic before P150, each study session at room temperature (24–26 °C) was limited to less than 15 min. Forelimb activity was recorded using a video camera. The animal was removed from the pouch and laid supine with the head and torso secured onto a V-shaped holder. The forelimbs were allowed to move freely. The video camera was placed about 50 cm above the animal. White markings were painted onto the wrists and the torso for measuring the relative forelimb position. For in-pouch activity, the pouch was opened gently and the pouch young (joey) moved towards the opening. Motor activity was videotaped while the joey remained attached to the teat. In some cases, the afferent inputs were altered by restricting the activity of one limb. For newborn wallabies, a silicone tube was put over the whole limb to restrict its movement. For animals older than P30, the forelimb was held down by hand. The effect of ambient temperature on limb activity was tested by placing the animal into an incubator fitted with a glass top so that limb activity could be monitored. To quantify the forelimb activity, the videotape was replayed and frame grabbed, and limb positions digitized manually. Plots of limb position *versus* time were made. Cubic splines were fitted (Fig. 1*B*) to these plots to assist the measurement of cycle frequency.

In vitro preparations

For *in vitro* studies, a total of thirty-five animals were used, with ages ranging from P3 to P42. The joey was first anaesthetized by hypothermia by placing it onto a dish filled with crushed ice. The animal was kept in a supine position until the cessation of rhythmic forelimb movement and spontaneous breathing. This took 1–2 min in most neonatal animals and less than 5 min with the oldest preparation (P42). The cerebral cortex and thalamus were immediately removed in a single cut through the soft cartilage using blunt dissection scissors; this was followed by evisceration while the animal remained in the ice. Dissection of the brainstem and spinal cord was then continued in superfused oxygenated isotonic sucrose solution cooled to about 10 °C. The composition of the sucrose solution was similar to the artificial cerebrospinal fluid (ACSF, for composition see below) except the NaCl was replaced with sucrose (266 mM). Any remaining midbrain was removed, up to the pontine flexure. Individual muscle nerves from the brachial outflow were freed. Routinely, nerves to the biceps brachii, triceps brachii, and subscapularis from both sides were dissected. In some situations, lumbar segmental nerves were also dissected for recording. The preparation was transferred to the recording chamber superfused with ACSF having the following composition (mM): NaCl, 133; KCl, 3; $NaHCO_3$, 17; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 12·2; oxygenated with 95% O_2–5% CO_2; pH 7·2–7·4. The bath temperature was maintained at 25 °C during recording.

Neural activity was recorded using a tight-fitting polyethylene suction electrode connected to a high gain DC amplifier (Grass P16). Wide bandwidth (DC–5 kHz) was used to record the slow electrotonic potential and propagated spike activity. Activity was displayed and recorded using the Axotape software (Axon Instruments, New York). Signals were also digitized (Neurocorder DR-890, Neuro Data Instruments) and recorded on videotape for further analysis. Integrated neurograms were constructed to compare the activity between muscle nerves and the effects of pharmacological agents. Selected segments of recorded signal were replayed and digitized at 5 kHz using the Axotape software. The digitized waveform was then filtered using the digital filter function of the Matlab software package (The MathWorks Inc., Natick, MA, USA) at a bandwidth of 10 Hz to 5 kHz to remove any slow DC drift. The baseline was then shifted to zero by subtracting the mean value of regions of the trace with no obvious neural activity. The waveform was then rectified. The integral of the trace was then computed with respect to a time duration of 200 or 400 ms using trapezoidal integration. The mean + 1 standard deviation (S.D.) of the integrated values from regions with no neural activity was used to determine the baseline of the integrated neurogram. For evoked activity, the region before stimulation was used. The integrated neurograms were also averaged from many cycles during spontaneous and evoked episodes to compare the phase relationship between different motor outflows. The trigger point for averaging was defined as the time when the peak discharge occurred (or in some cases the onset of discharge) in the designated nerve during a cycle. A 10 s time window was used for averaging, with a 2–4 s pretrigger period. The integrated neurograms from other nerves within an averaging time window were normalized to the peak amplitude of the designated nerve.

Pharmacological agents were prepared as 10–50 mM stock solutions in distilled water and kept frozen until use. An appropriate amount was added directly to the ACSF circulating in the recording chamber. *N*-Methyl-D-aspartic acid (NMDA), (−)-bicuculline methbromide, strychnine hydrochloride and picrotoxin were all purchased from Research Biochemicals International.

RESULTS

Behavioural studies

Wallaby pouch young displayed a remarkable clock-like forelimb movement when removed from the pouch. This movement normally continued throughout an entire behavioural study session, which lasted about 10–15 min. The rhythmic motor activity was also characterized by a tight alternating pattern between contralateral limbs as illustrated in Fig. 1. When one limb was extended to the most rostral position, the contralateral limb was retracted. In-phase activity between both limbs was never observed. In newborn animals, the air stepping frequency was lower, about 0·1–0·2 Hz. Missing cycles, where limb movement failed to occur, were observed in nine out of ten animals between P1 and P3 (see Fig. 1, P1). The stepping frequency became progressively faster and the pattern more regular as the animal matured. Figure 2*A* summarizes the increase in stepping frequency with development from twenty-nine animals between P1 and P42. It levels off at about 0·4 Hz by the 4th postnatal week. By grouping the mean cycle frequency obtained from each preparation into four age groups, P1–P7, P8–P14, P15–P21, >P22, it was found that there was a significant increase in cycle frequency with age (using ANOVA, $F = 24{\cdot}7$, degrees of freedom (d.f.) = 3,29, $P < 0{\cdot}001$). The phase relationship between contralateral limbs remained around 0·5 (Fig. 2*B*). Though a larger scatter in phase value was observed in the younger age group, ANOVA failed to show any significant change with age ($F = 0{\cdot}1$, d.f. = 3,25, $P > 0{\cdot}95$). Forelimb activity persisted in most pouch young until the time of eye opening, which occurs around P140. At this time, the forelimbs are actively used to grab onto surrounding objects in order to maintain balance whenever the animal is removed from the pouch.

When the joey was inside the pouch and remained attached to the teat, forelimb activity was sparse. Figure 3 shows two examples of limb activity obtained by videotaping the pouch young through the opening of the pouch. In contrast to the continuous stepping activity when the animal was removed from the pouch, forelimb activity was only observed after mechanical arousal by stroking the skin of

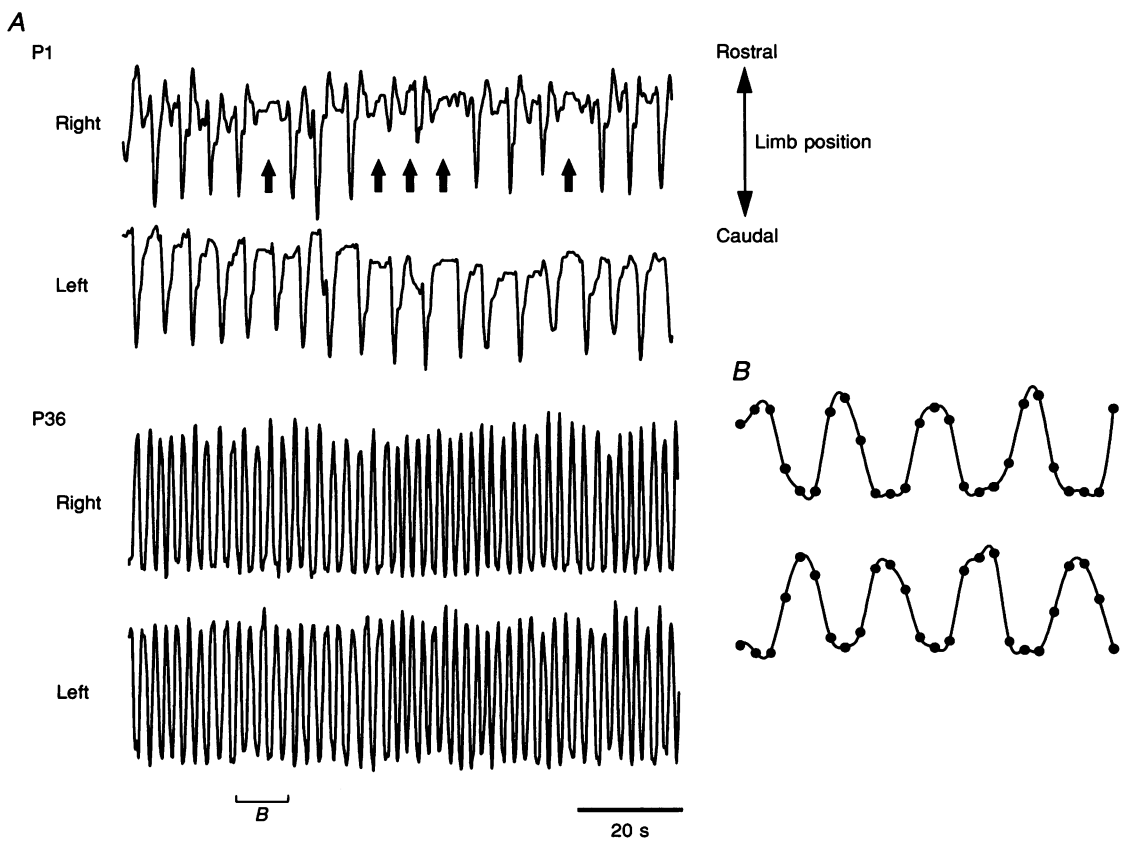

Figure 1. Forelimb air stepping activity in P1 and P36 pouch young

A, the traces representing limb position digitized from video records. Most animals showed continuous activity when removed from the pouch. *B*, enlargement of region indicated in *A*. A tight alternating phase relationship was maintained (as shown in *B*), especially in older animals. In the P1 trace, stepping frequency was slower and less regular. This example shows missing cycles from the right limb (arrows). The filled circles in *B* represent the digitized limb positions. These are joined with a cubic spline.

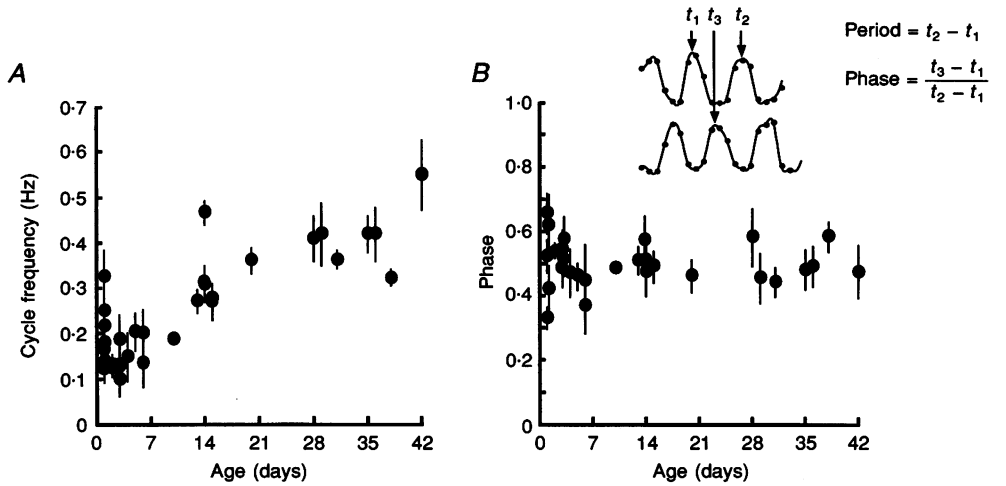

Figure 2. Summary of cycle frequency (*A*) and phase relationship (*B*) during in-pouch development

There was a progressive increase in forelimb cycle frequency during the first three postnatal weeks. The alternating pattern between contralateral limbs remained unchanged during this period. The inset indicates how cycle frequency and phase were determined from the digitized records. Each symbol and bar represent the mean and S.D. averaged from at least fifty cycles of activity measured in each animal.

the back or blowing into the pouch. This evoked forelimb activity diminished after about ten to fifteen step cycles. It was difficult to measure the alternating activity from both limbs as the angle of the video camera was restricted and normally only one side of the animal was in view. However, from casual observation, the forelimbs moved in an alternating fashion when activated, similar to ex-pouch air stepping.

The behavioural studies showed the forelimb locomotor ability, which is crucial for the newborn to climb into its mother's pouch, was not eliminated after the first journey. However, the robust forelimb movement was observed only when the animal was removed from the pouch. This suggests that other sensory inputs are involved in controlling this motor behaviour. To investigate this further, one possible source of sensory input was manipulated by altering the afferent feedback from the contralateral limb. This was achieved by restraining contralateral limb movement during air stepping at room temperature. Figure 4 illustrates the effect of such manipulation from a joey on P28. The onset of a step cycle was delayed when movement from the contralateral limb was stopped. This was especially prominent during the beginning of the perturbation. By measuring the time required for the first

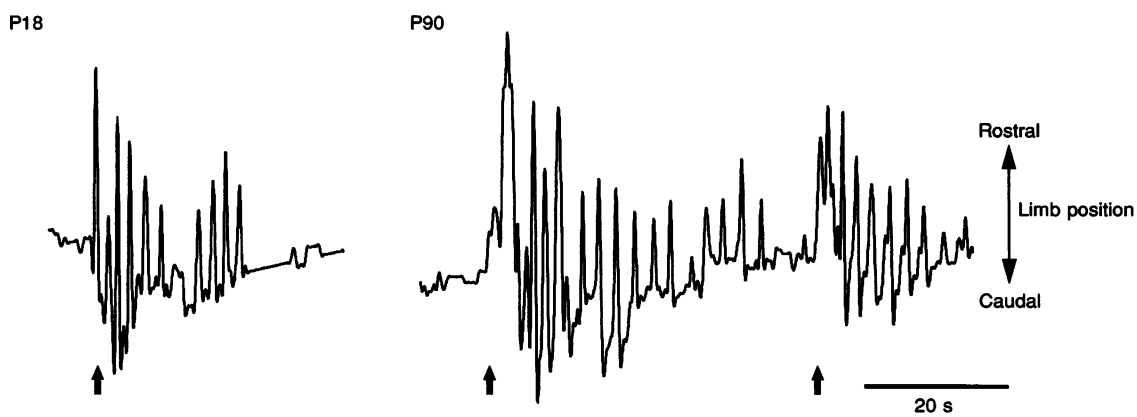

Figure 3. In-pouch forelimb activity

The two traces show forelimb activity from P18 and P90 recorded with the joey inside the pouch. In contrast to ex-pouch movement, forelimb activity was sparse when the animal was inside the pouch and remained attached to the teat. Upon mechanical arousal (arrows) an episode of movement, consisting of around ten cycles of limb activity, could be observed.

five steps immediately after the pertubation, it was found that the reduction in step cycle frequency was about 30–40% for animals older than 3 weeks (Fig. 4*B*). It was unlikely that muscle afferent activity was eliminated by restraining the limb movement. Furthermore, additional cutaneous feedback may be initiated when the limb tried to move against the restrainer. However, the slowing down of limb movement suggests the contralateral afferent feedback plays a role in governing the rhythmic forelimb activity. This retarding effect was less effective in younger animals (Fig. 4*B*). By dividing the data into two groups, P1–P14 and >P14, the more mature group showed a significant reduction compared with the younger group (Student's t test, $P < 0{\cdot}05$). This implies that the afferent pathway has less influence on the rhythmic motor activity during early development.

The effect of temperature was tested in twelve animals (divided into 3 age groups) by measuring the change in air stepping activity inside a humidified incubator with the temperature set at 37 °C, similar to the in-pouch condition. Forelimb activity in older animals was suppressed in both amplitude and frequency. This was especially effective in animals older than P90. A complete cessation of forelimb movement could be observed within 3–5 min of the increase in temperature (Fig. 5, P95). Conversely, a drop in temperature from 37 °C back to room temperature immediately elicited forelimb activity. In younger animals, an initial reduction in stepping frequency of 20–30% was observed during the first few minutes of the increase in temperature (Fig. 5*B*). While the activity in P40–P90 animals came to a halt after 5 min, this did not happen to animals younger than P40.

The well developed pronated forelimbs are a prominent feature of newborn wallabies. The epitrichial claws are used to gather and clasp the fur during the first climb into the pouch. Co-ordination between forelimb movement and digito-palmar prehension could be observed during air stepping activity. During a step cycle, digits were extended when the limb was protracting. The paw was then closed during arm flexion and adduction (Fig. 6*B*). The effect of increasing ambient temperature on forelimb and digito-palmar activities is illustrated in Fig. 6. In this preparation, movement of the right limb was reduced shortly after increasing the ambient temperature to 37 °C, although at this stage the left limb continued to move in a rhythmic manner (arrow in Fig. 6). Furthermore when movement from both limbs was halted, activity of the paws remained alternating (Fig. 6*C*). Dissociation between forelimb movement and digito-palmar prehension was observed in three out of four animals in the > P90 group, one out of four in the P40–P90 group. In the youngest group, limb activity remained co-ordinated 5 min after increasing the temperature to 37 °C. This suggests the co-ordinated movement between contralateral limbs and digito-palmar

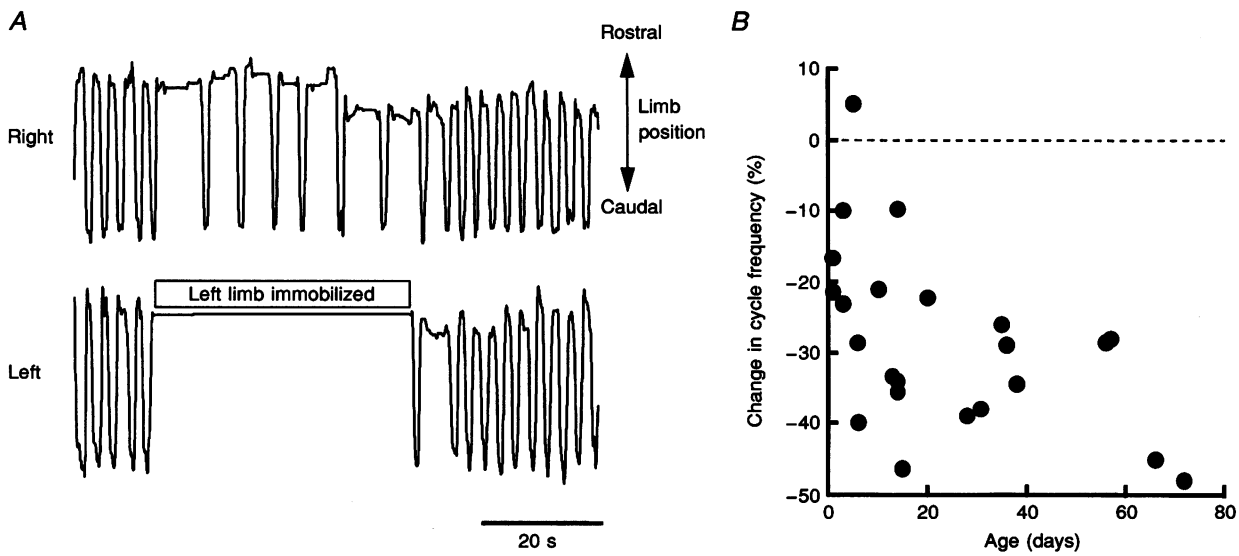

Figure 4. Decrease in limb activity after altering contralateral afferent inputs

A, limb position traces showing the change in limb activity after restraining the contralateral limb. The onset of limb movement was delayed, especially during the beginning of the immobilization. The cycle frequency during this period was determined by measuring the time required for the first five steps immediately after contralateral limb immobilization. This was compared with cycle frequency before and after the perturbation of limb movement. *B*, summarizes the reduction in cycle frequency in animals of different ages. The reduction in limb activity is less apparent in younger animals suggesting the afferent pathway has less influence on the rhythmic motor behaviour during early development. Each symbol represents an individual animal.

prehension can be disengaged from one another under different situations. This ability may enable different motor pools to be selected to perform different motor tasks. The co-ordinated activity between the forelimbs and the claws is important during the first climb to the pouch. The rhythmic palmar activity may also play a crucial role in providing mechanical stimulation of the mammary gland as sensory feedback from the pouch can influence lactation and the hormonal control of the mother's reproduction cycle (Renfree, 1979).

Isolated brainstem–spinal cord preparation

Another goal of the present study was to determine the feasibility of utilizing the wallaby preparation under *in*

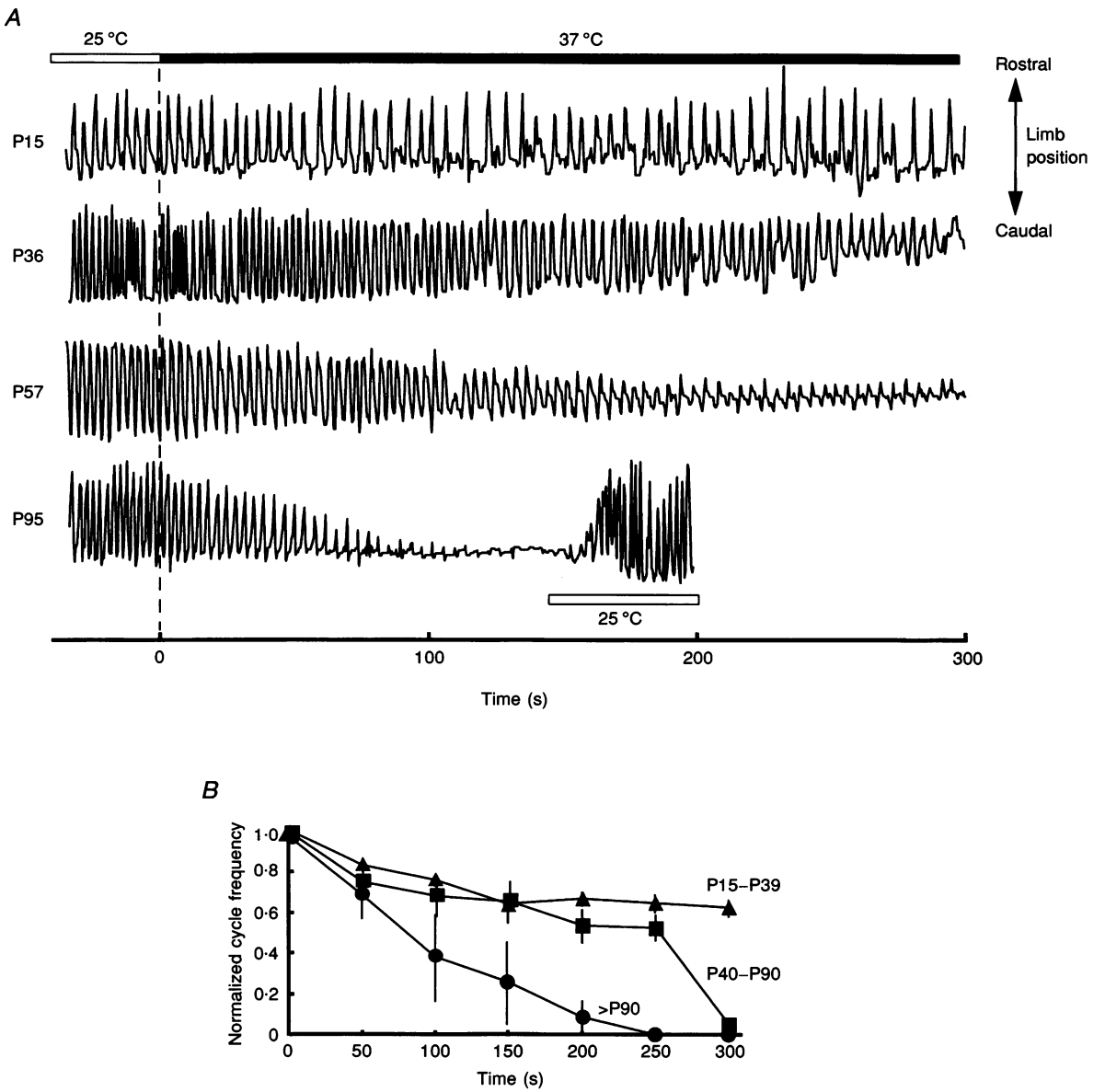

Figure 5. Change in forelimb activity after increasing the ambient temperature

A shows single forelimb position traces from four animals. After recording the control activity at 25 °C, the animal was placed inside an incubator (time = 0). Forelimb activity in older animals was suppressed in both amplitude and frequency. This was especially effective in animals older than P90, bringing a complete halt to limb movement within 3–5 min. The P95 animal was returned to room temperature, as indicated by the open bar, and forelimb activity was reactivated immediately. This reactivated forelimb activity immediately. *B* summarizes the change in instantaneous limb frequency normalized to the value before the temperature increase. Unlike the other two more mature groups where limb activity was diminished after 5 min, rhythmic forelimb movement from P15–P39 animals remained, though frequency was reduced by about 30%. Each group contains results from four animals. Each symbol and bar represents mean and S.E.M., respectively. All preparations in the >P90 group showed a reduction in frequency before the ceasing of limb movement. The larger error bars were due to different onset of reduction.

vitro conditions. After the initial dissection, the preparation was kept in normal ACSF at 20–21 °C for 4–6 h before warming the bath to 25 °C. In six preparations (17%), no rhythmic response could be evoked by direct stimulation or the use of bath-applied excitatory transmitter. Out of the remaining preparations, about half (12/29) expressed spontaneous motor activity, which occurred once every 2 min or longer, in control ACSF after increasing the bath temperature. All preparations remained viable throughout the experiment, which lasted about 12 h, provided continuous motor activity was not induced with bath-applied agonists. Six preparations were deliberately kept overnight. All of them, including a preparation from a P42 animal, continued to show rhythmic activity after 30 h in the bath.

Figure 7*A* shows the activity of muscle nerves recorded from a P19 preparation during an evoked episode of fictive locomotion. A single pulse stimulation (square current pulse, 0·7 mA, 0·3 ms duration) was applied to the dorsal part of the brainstem using a suction electrode. In control ACSF, the number of cycles in each evoked episode was 7·7 ± 1·3 (averaged from 3 episodes). This was increased to 13·6 ± 1·5 (averaged from 6 episodes) when 1 μM NMDA was added to the bath. Moving the stimulation electrode to the dorsal thoracic cord could also elicit rhythmic activity. Using the same stimulation intensity, the number of cycles per evoked episode was 11·6 ± 2·2 (averaged from 6 episodes). Figure 7*B* shows the change in cycle frequency measured from the same preparation during thoracic cord-evoked episodes. The range of cycle frequency varied between 0·5 and 0·2 Hz, becoming slower towards the end of the episode. This frequency range was comparable to the natural limb movement observed during behavioural studies (compare with Fig. 2). Figure 7*C* shows the phase relationship between contralateral triceps, and ipsilateral biceps and triceps nerves. The motor discharge was co-ordinated: alternation between contralateral nerves, and phase shifting between ipsilateral flexor and extensor. Spontaneous motor activity could also be induced by increasing the NMDA concentration. The dose response of excitatory transmitter on cycle frequency was not examined in detail in the present study. In general, when the concentration of bath-applied NMDA first reached a sufficient level to evoke spontaneous activity, the firing pattern of motor pools was characterized by bouts of discharge, each consisting of two to three bursts of firing. This discharge pattern is illustrated in Fig. 7*D*, which was obtained from a P27 preparation with 3 μM NMDA added

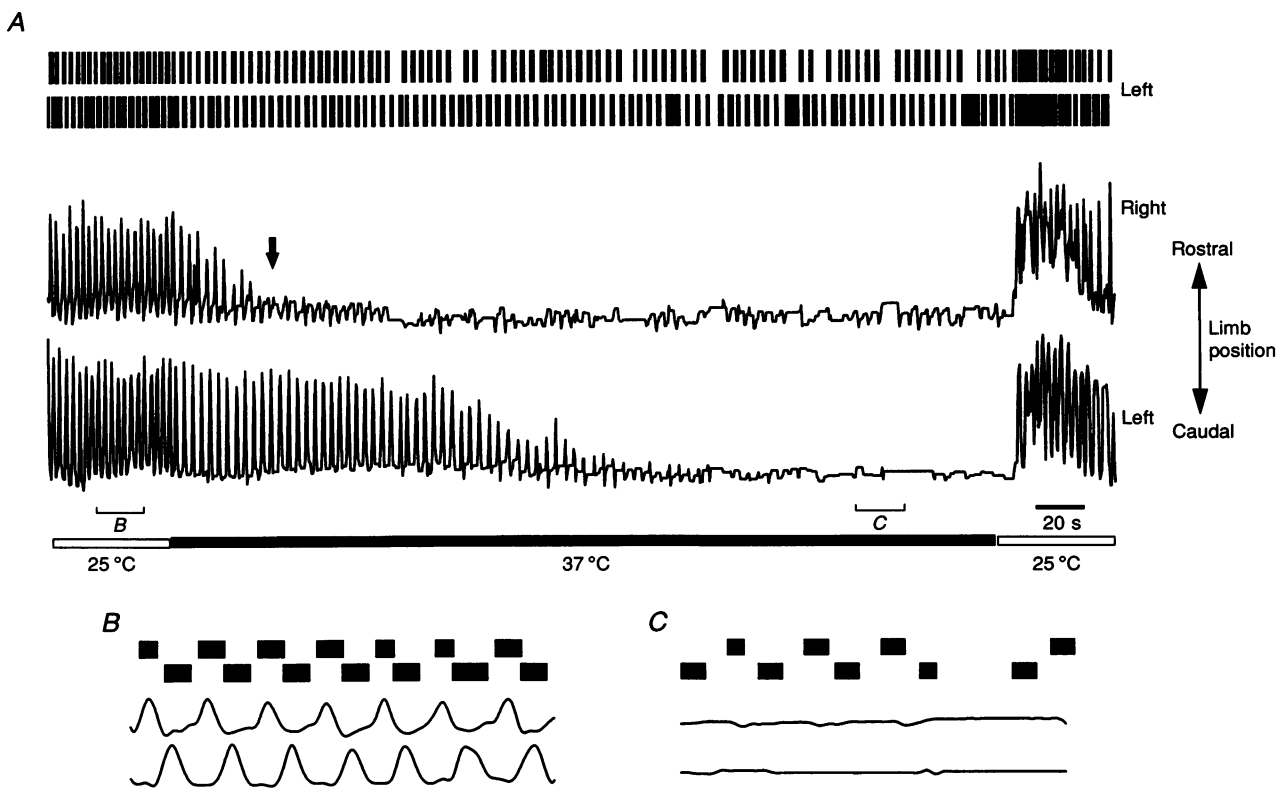

Figure 6. Differential effect of temperature on forelimb and digito-palmar activities

A, filled bars in the top two traces indicate the digits are in an extended position. The third and fourth traces represent forelimb position. Digits are normally extended during the protraction of the forelimb. The paw is then closed when the arm flexes and adducts. *B* shows this relationship on an expanded time scale. When the ambient temperature was raised, the forelimb activity reduced progressively while the alternating digito-palmar prehension remained (*C* shows this on an expanded time scale). *B* and *C* are enlargements of the corresponding regions indicated in *A*.

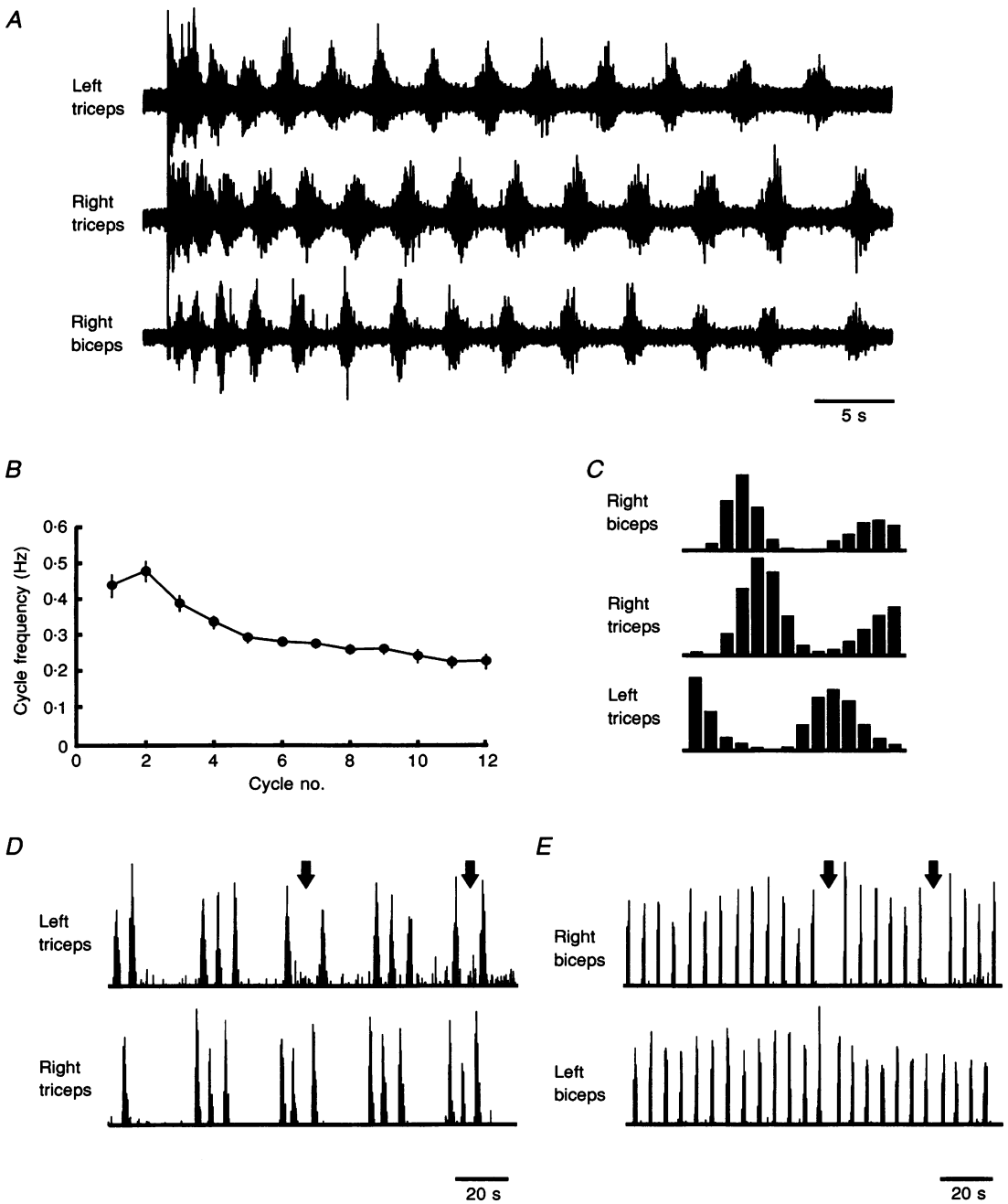

Figure 7. Co-ordinated motor discharge recorded from an isolated brainstem–spinal cord preparation

A, muscle–nerve recordings showing an episode of motor activity evoked by a single pulse stimulation applied to the thoracic cord in a P19 preparation. The traces were bandpass filtered at 10 Hz to 5 kHz digitally. The ACSF contained 1 μM NMDA. There was a progressive reduction in cycle frequency during evoked episodes. Each symbol and bar in *B* represents mean and S.D. of cycle frequency averaged from ten episodes. Alternation between contralateral nerves and phase shift between ipsilateral flexor and extensor is illustrated in *C*. Each histogram represents the mean integrated neuroactivity of the corresponding nerve averaged from thirty-eight cycles of discharge. Each bin represents total activity during a 400 ms period. Cycles were aligned for averaging by the onset of the right triceps discharge. The amplitude of activity in each cycle was normalized to the peak of the right triceps discharge. Cycles at the beginning of the episode where activity was fused were not included in the averaging. *D*, appearance of spontaneous motor activity in a P27 preparation when bath NMDA level was increased to 3 μM. *E* illustrates the spontaneous motor activity becoming more regular when the NMDA level was increased to 7 μM in a P3 preparation. 'Missed cycles' could be observed in both ages (arrows in *D* and *E*). Both *D* and *E* are integrated neurograms with an integration time of 400 ms.

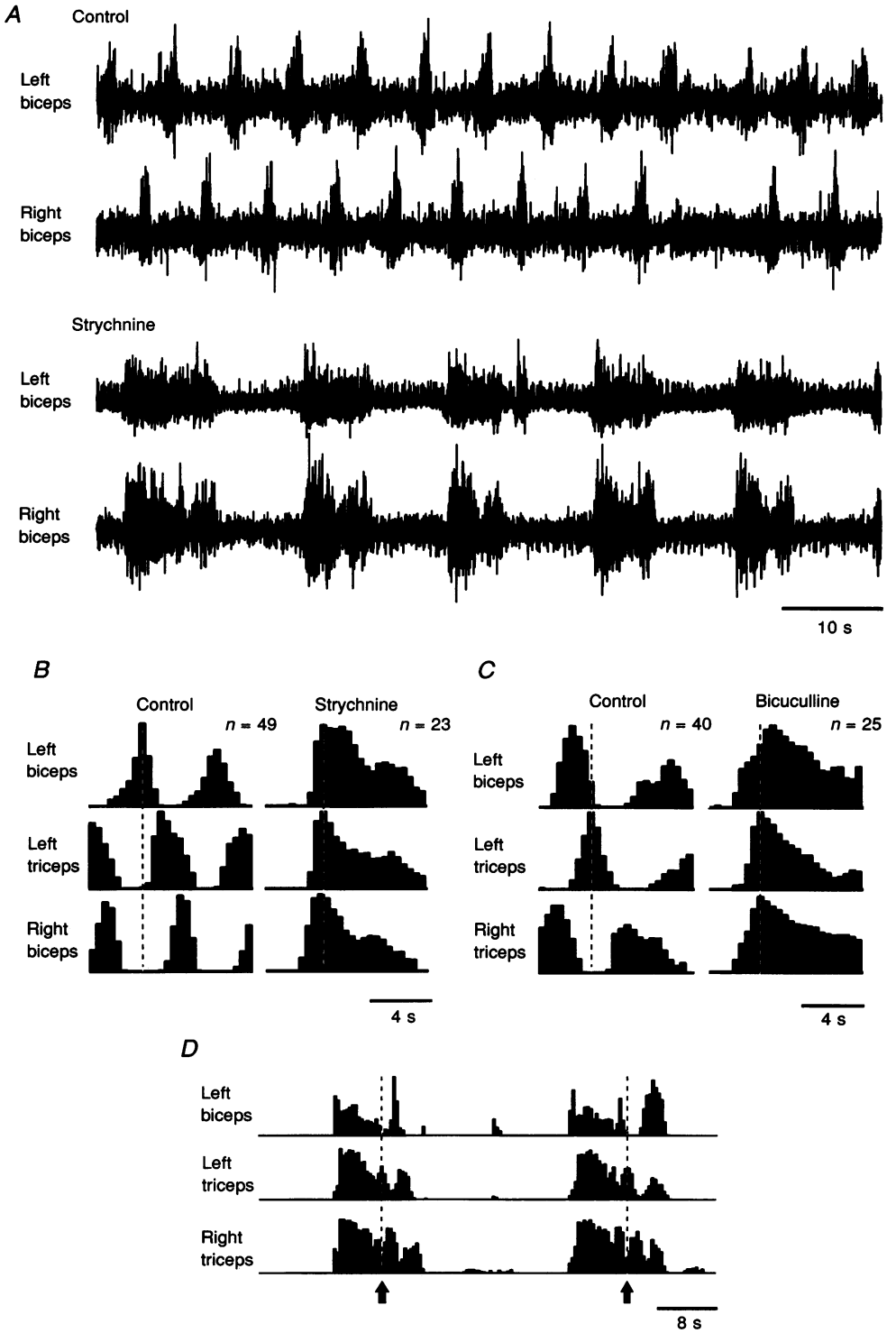

Figure 8. Effect of glycine and $GABA_A$ antagonists on the pattern of motor discharge

A, the alternating activity between contralateral nerves was changed to coactivation in a P3 preparation after bath application of strychnine (25 μM). Bath contained 7 μM NMDA. Histograms in *B* represent averaged integrated neurograms from this preparation while those in *C* show the change in discharge pattern after applying 10 μM bicuculline in another P19 preparation. An integration time of 400 ms was used in both cases and the number of cycles used for averaging is indicated in the top right. Cycles were aligned by the peak discharge of the left biceps and left triceps for *B* and *C*, respectively (dashed lines). *D* illustrates the neurograms of two consecutive bursts from the same preparation shown in *C*. Although all three nerves had similar onset, phase separation was evident towards the end of the burst (arrows). As the burst duration varied between cycles, these differences were smeared out in the averaged neurograms.

to the ACSF. Figure 7*E* illustrates a more regular motor pattern in a P3 preparation with a higher concentration of NMDA (7 μM). These records were selected to show the occurrence of 'missing cycles' (arrows in *D* and *E*), which could also be observed in the behavioural studies (see Fig. 1). With higher concentration of NMDA (>10 μM), tonic activity occurred.

The effect of the glycinergic pathway on the pattern of motor discharge was examined. Application of strychnine led to a period of enhanced motor discharge and tonic firing lasting about 10–20 min. This was followed by a quiescent state, or rhythmic bursting if NMDA was added. Recordings were made 30 min after the application of strychnine where tonic activity had subsided. Figure 8*A* illustrates the change in motor discharge profile in a P3 preparation after bath-applied strychnine (25 μM). The histograms in Fig. 8*B* show the averaged integrated motor discharge recorded from the same preparation. The alternation between left–right (L–R) biceps and phase shift between ipsilateral biceps and triceps were disrupted after blocking glycinergic activity. The firing from all three nerves had a similar onset, and the discharge duration was lengthened. All preparations (P3–P42) tested for the effect of strychnine (10–25 μM) showed coactivation between L–R nerve pairs ($n = 4$) or a pair of ipsilateral antagonistic nerves ($n = 3$).

Figure 8*C* and *D* shows a similar conversion from alternation to coactivation after the addition of bicuculline (10 μM), a $GABA_A$ antagonist. It is important to point out that remnants of alternation or phase separation could be observed in some cycles, especially towards the end of the burst. In Fig. 8*D*, two consecutive bursts showing such properties are illustrated. The integrated neural activity is displayed as histograms, with each bin representing activity within a 400 ms period. Although all three nerves showed a similar onset of firing, variation in their discharge intensity could be seen towards the end of the bursts. As indicated by the arrows in Fig. 8*D*, the increase in the left triceps activity was accompanied by a reduction in activity in the contralateral nerves. Likewise, a phase shift appeared between ipsilateral biceps and triceps. This pattern of modulation was also observed after the application of strychnine (data not shown). A lengthening of burst duration and coactivation between L–R nerve pairs was observed in three other preparations after the addition of 10–50 μM bicuculline. Picrotoxin (100 μM) was used in another preparation and a similar conversion from alternation to coactivation was

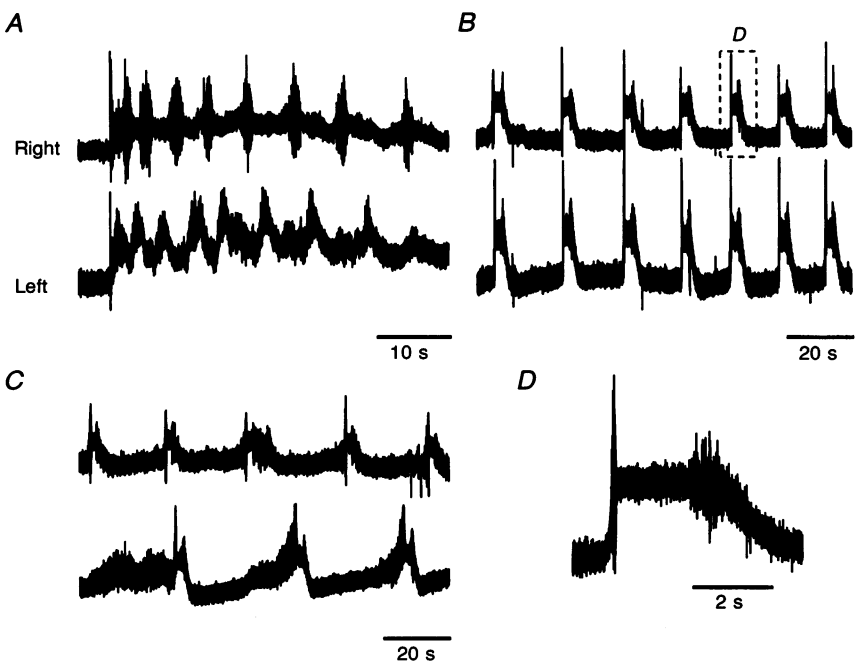

Figure 9. Combined effect of bicuculline and strychnine on the pattern of motor discharge

A, control recordings from the left and right subscapularis nerves during an evoked episode from a P24 preparation. To show the slow electrotonic potential, as well as the propagated spike activity, neurograms were not high-pass filtered. Bath NMDA concentration was 2 μM. *B*, addition of strychnine (25 μM) and bicuculline (50 μM) changed the alternating activity into coactivation. Each cycle was initiated with a strong burst of motor discharge, accompanied by depolarization. A pause in motoneurone firing appeared although depolarization was maintained during this period (see enlargement shown in *D*). Motor discharge resumed towards the end of the cycle. *C*, spontaneous rhythmic bursting can be generated in a hemisected brachial cord. The neurograms show activity from the preparation shown in *B* after hemisection and separation from the brainstem by transecting at C1–C2 level. The cycle frequency was reduced and the onset became less distinct. Bath NMDA was 4 μM for *B* and *C*. *D*, enlargement of region indicated by box in *B*.

observed. Lower concentration of bicuculline (5 μM) was also tested in three preparations. Only one of them showed a change to coactivation.

Cycle frequency was also affected by strychnine and bicuculline. A significant increase (Student's t test, $P < 0{\cdot}5$) in cycle frequency was observed in two out of three preparations with 5 μM bicuculline. Cycle frequency was averaged from at least 20 cycles in control and after application of the antagonist. However with a higher concentration of bicuculline (10–50 μM), all four preparations showed a significant reduction in cycle frequency. This was averaged to 26% of the control value. With strychnine (10–25 μM), the cycle frequency averaged from six preparations was reduced to 88% of control, and only three of them showed a significant reduction. The measurement of cycle duration was affected by discharges that appeared in close sucession (Fig. 8*D*) towards the end of a cycle. The averaged cycle duration was shortened when these discharges appeared frequently since a burst was counted as an individual cycle when the preceding activity dropped by more than 25% of the previous peak. This criterion, though arbitrary, was necessary as some cycles appeared without complete cessation of activity in between (see Fig. 7*A*).

In another set of experiments strychnine (25 μM) and bicuculline (50 μM) were applied together. Similar to the results described in the previous section, the pattern of activity was dominated by the synchronized onset of discharge between contralateral nerves and ipsilateral flexor and extensor. The important difference was the appearance of a biphasic discharge (see Fig. 9*D*). This type of response was observed in seven out of nine preparations. On the other hand, this type of discharge pattern was only observed in three out of thirteen preparations when either strychnine or bicuculline was applied alone. Wide bandwidth (DC–5 kHz) recordings are shown in Fig. 9 to illustrate the slow electrotonic potential recorded from the muscle nerves. Due to the short distance between the recording site and motoneurones (2–3 mm), the slow potential reflects the aggregate change in intracellular potential of the motor pool (O'Donovan, 1989). During each cycle of activity, motoneurones were depolarized during the entire cycle, but firing was restricted to the beginning and the end of the cycle. Furthermore, the presence of these antagonists did not prevent the expression of rhythmic activity. Figure 9*B* shows the spontaneous rhythmic discharge, now synchronized between contralateral nerves, when the bath NMDA was increased to 4 μM. This coactivating pattern remained when the brainstem was removed (observed in 4 preparations, data not shown) by transecting the cord at the C1–C2 level, suggesting the presence of mutual excitation between contralateral hemicords at the segmental level. Rhythmic discharge persisted in a hemisected brachial cord (C2–T2). The cycle frequency was slower and the onset of motor discharge less distinct (Fig. 8*C*). This was repeated in another preparation though cyclic activity could only be recorded in one of the hemicords.

The hindlimbs have large developmental differences compared with the forelimbs. At birth, the hindlimbs exist as limb buds while the forelimbs are capable of functional movement. Even at 4–5 weeks postnatal, there is no active movement from the hindlimbs, although they are capable of withdrawal reflexes (personal observation). In the last part of this study, the ability of the lumbar spinal cord to generate rhythmic motor activity was tested. This was carried out in three preparations, which were used for studying the effect of strychnine and bicuculline on the brachial motor pattern. Figure 10 illustrates the wide bandwidth (DC to 5 kHz) neurograms recorded from the brachial subscapularis nerve and the 6th lumbar segmental nerve (L6) of a P27 preparation in control ACSF. These nerves were chosen to reflect the activity in the brachial and lumbar cords. During a single pulse (square current pulse, 0·3 mA, 0·3 ms duration) evoked episode, the brachial cord showed many cycles of activity while the lumbar gave only a few bursts of discharge. In the presence of strychnine (25 μM) and bicuculline (50 μM), spontaneous rhythmic activity could be ellicidated from the isolated lumbar cord

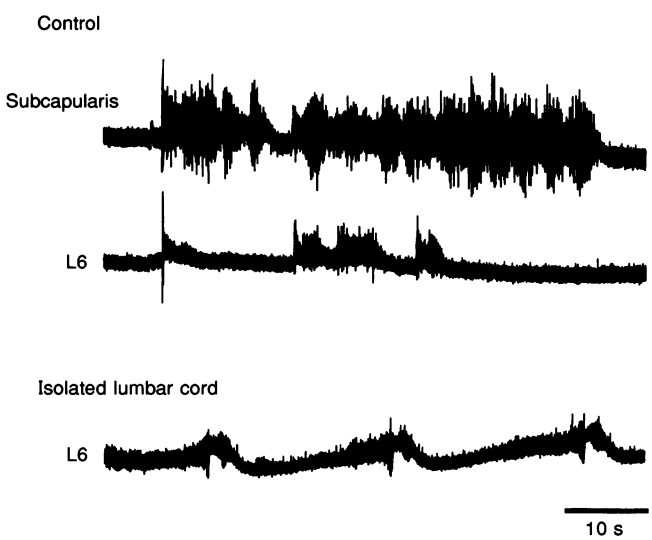

Figure 10. Different efficacies in generating rhythmic activity in the brachial and lumbar cords of a P27 preparation

The upper panel shows neurograms recorded from the subscapularis and the ipsilateral 6th lumbar segmental nerves during a brainstem evoked episode. The brachial cord showed more intense activity than the lumbar. No pharmacological agent was added to the bath at this stage. The lower panel shows activity from the same preparation after isolating the lumbar cord by transecting the cord at T11–T12 level. Spontaneous activity appeared when bath NMDA level was increased to 5 μM. The bath also contained strychnine (25 μM) and bicuculline (50 μM).

(T12–L7) with bath-applied NMDA (5 μM). The lower panel of Fig. 10 shows rhythmic motor discharge recorded in an isolated lumbar cord (T12–L7). The activity was characterized by a gentle ramp of depolarization preceding each burst, followed by repolarization. Similar activity was observed in the other two preparations. This pattern was similar to those expressed in an isolated and hemisected brachial cord (compare with Fig. 9*C*).

DISCUSSION

This study is the first to document the forelimb behaviour of tammar wallabies during in-pouch development. It demonstrates that such patterned activity can be generated in an isolated brainstem–spinal cord preparation. The data also show the existence of mutual excitation between contralateral hemicords and both glycinergic and GABAergic pathways are required to maintain intra- and interlimb co-ordination.

The ability to generate rhythmic forelimb movement is not lost after the joey has entered the pouch. This stereotyped movement can be reactivated by changing the somatosensory inputs. The 'missing cycles' seen in neonates during the behavioural studies were seldom observed in older preparations, and cycle frequency almost doubled after the first two postnatal weeks (Fig. 1). This improvement in limb motility is comparable to that seen in neonatal rats where cycle duration reduced by more than 50% during the first two postnatal weeks (Bekoff & Trainer, 1979; Cazalets, Menard, Cremieux & Clarac, 1990). Our preliminary data demonstrate that growth cones of primary afferents have just reached the ventral brachial cord at birth, and the static and dynamic components of spindle response became distinguishable only after 4 weeks postnatal (R. V. Stirling & S. M. Ho, unpublished observation). The results in Fig. 3 demonstrate that rhythmic activity can be affected by afferent input in an age-dependent manner. This suggests that the improved rhythmicity is in part due to the establishment of muscle afferents. This agrees with other findings where the operation of the spinal CPG could be modulated or entrained by other inputs including sensory information from muscle afferents (Bekoff, Nusbaum, Sabichi & Clifford, 1987; Pearson, Ramirez & Jiang, 1992; Kiehn, Iizuka & Kudo, 1992; Kriellaars, Brownstone, Noga & Jordan, 1994).

Besides the important role during the first climb from the birth canal to the pouch, a report by New, Mizell & Cockroft (1977) suggests the preparturient onset of locomotor movements can cause rupture of the extra-embryonic membranes and form part of the birth-initiating complex. The demand of the spinal CPG subserving locomotor function becomes episodic after pouch entry. Pouch young by P70 are known to detach intermittently from the teat and move around the pouch (L. Marotte, personal communication). Another possible function of the spinal CPG is to provide stimulation to the mammary gland, which is crucial in maintaining the hormonal balance of the mother's reproductive cycle. The female wallaby carries a dormant embryo when nursing a joey in the pouch. Local denervation of the suckled mammary gland causes reactivation of the diapausing blastocyst (Renfree, 1979). As the claws are normally positioned near the base of the teat, their rhythmic activity would provide focal mechanical stimulation. All these indicate the output of the spinal CPG can be modulated to serve in a different capacity as the needs of the animal changes during in-pouch development.

As the firing pattern of muscle groups varies during different behaviour, the 'unit burst generator' was introduced to explain the organization of synaptic drives to a motor pool (Grillner, 1981). Each unit generator or module is sufficient to produce rhythmic activity and different patterns of activity arise depending on the interactions between the modules. Stein and his group have demonstrated the selective activation of individual modules that control the hip extensor and flexor during fictive scratching (Stein, Victor, Field & Currie, 1995). The tammar wallaby provides another opportunity for studying this hypothesis. The disruption of inter- and intralimb co-ordination (Fig. 5) can be used to examine whether proximal and distal muscle groups in each limb are controlled by separate modules and the change in temperature causes activity in some modules to cease. Alternatively, some motor pools may disconnect or 'drop out' from the on-going rhythmic drive. On some occasions, a motor pool could skip a cycle of discharge during rhythmic episodes (Fig. 6*D* and *E*). Furthermore, coactivation between left and right forelimbs was never observed during the transition period of temperature increase. All these suggest the two contralateral modules maintain a tight interaction but fail occasionally to drive some of the motor pools. Further experiments are needed to investigate factors that govern the coupling between the CPG and motor pools.

The glycinergic inhibitory pathway plays an important role in many spinal networks responsible for rhythmic motor behaviour. Extensive studies in both lamprey and *Xenopus* embryo show glycinergic inhibition is the predominant pathway that maintains left–right alternation (Buchanan, 1982; Cohen & Harris-Warrick, 1984; Soffe, 1987; Alford & Williams, 1989). Glycine is also involved in controlling the alternation between contralateral limbs (Kudo, Ozaki & Yamada, 1991; Cowley & Schmidt, 1995), and intralimb flexor and extensor (Pratt & Jordan, 1987) in higher vertebrates. In agreement with these findings, bath-applied strychnine disrupted the alternation between contralateral motor pools and the phase separation between ipsilateral flexor and extensor (Fig. 8*A*), suggesting the glycinergic pathway is also involved in inter- and intralimb co-ordination in the tammar wallaby.

$GABA_A$ inhibition plays a dominating role in establishing intralimb co-ordination in the embryonic chick spinal cord (Sernagor, Chub, Ritter & O'Donovan, 1995). In the present study, bicuculline (10–50 μM) perturbed the alternating

pattern in the wallaby spinal cord (Fig. 8C). This is consistent with the report by Cowley & Schmidt (1995), who showed the involvement of both glycinergic and GABAergic (acting through $GABA_A$ receptor) inhibition in establishing the contralateral alternation in the neonatal rat preparation. The present results differ from those reported by Cazalets *et al.* (1994), where bicuculline enhanced the cycle frequency but failed to disrupt the contralateral alternation and organization between ipsilateral flexor–extensor even at concentrations up to 10 μM. Though other non-specific effects cannot be ruled out, it is unlikely that the dosage of bicuculline used in the present study would affect the glycinergic pathway. Glycinergic responses in motoneurones and hippocampal neurones persist in the presence of 50 μM bicuculline (Ito & Cherubini, 1991; Wu, Ziskind-Conhaim & Sweet, 1992). Alternatively, the increase in cycle frequency and the breakdown of left–right alternation may involve different $GABA_A$ receptor subtypes having a different affinity for the antagonist. Seven $GABA_A$ receptor subtypes, with distinct distribution, have been described in the rat spinal cord (Bohlhalter, Weinmann, Mohler & Fritschy, 1996). Different dose responses to $GABA_A$ antagonists for inhibiting antinociceptive effects also suggest that different $GABA_A$ receptors are involved in the spinal circuitry (Nadeson & Goodchild, 1994). Further experiments are required to clarify this issue.

Rhythmic activation of motor pools was not disrupted after bath application of strychnine and bicuculline in the wallaby spinal cord (Fig. 9). The blockade of inhibitory transmission further reveals the mutual excitation between contralateral brachial cords. Cord hemisection at this stage reduced the cycle frequency and the onset of motor discharge became less abrupt (Fig. 9C). Such conversion from a normally alternating pattern to a rhythmic synchronous discharge has been reported in the chick and rat spinal preparations after the application of strychnine and bicuculline (Sernagor *et al.* 1995; Cowley & Schmidt, 1995; Bracci *et al.* 1996). These confirm that rhythmic motor discharge can be generated in the absence of glycinergic and $GABA_A$ inhibition. It is also interesting to note that coactivation is first manifested during early development in both the rat and chick preparation. In the rat, simultaneous discharge was observed in the left and right ventral roots before E17 (Kudo *et al.* 1991). Similarly, coactivation of flexor and extensor was recorded in the chick embryo before stage 30 (O'Donovan & Landmesser, 1987). Subsequent establishment of inhibitory connections changes the activity pattern to alternation. It remains to be tested whether such a sequence also occurs in the prenatal wallaby brachial cord.

The developmental difference between fore- and hindlimbs in the wallaby provides another opportunity to examine spinal networks with different efficacy and maturity within the same animal. Although hindlimb movement was not observed after 5 weeks postnatal, the present study showed that the lumbar cord is capable of generating rhythmic activity. The number of cycles during an evoked episode was much lower than the brachial cord (Fig. 10). With the assistance of a bath-applied excitatory agent, an isolated lumbar cord can manifest rhythmic discharge that resembles activity expressed in a hemisected brachial cord. Whether it is the lack of contralateral connections or other intrinsic properties that result in a lower rhythmicity in the lumbar cord will be addressed in subsequent studies. It will also be interesting to determine whether the lumbar cord is capable of generating left–right alternation during development since adult tammar wallabies mainly utilize their hindlimbs in coactivation. Morphological differences between brachial and lumbar cord may further assist the identification of circuits that underlie the different functions.

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